Rec'd PCT/PTO 22 JUN2001

FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	210190US0PCT
	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR) 09/857213

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP99/07106	17 DECEMBER 1999	22 DECEMBER 1998

TITLE OF INVENTION

MONOCLONAL ANTIBODY AGAINST APOLIPOPROTEIN A-I

APPLICANT(S) FOR DO/EO/US

Osamu MIYAZAKI, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☒ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Request for Consideration of Documents in International Search Report
Notice of Priority / PCT/IB/304 / PCT/IB/308 / Drawings (8 sheets)
Amended Sheets (page 21)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/857213	PCT/JP99/07106	210190US0PCT

The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :	CALCULATIONS PTO USE ONLY
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1000.00	
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $860.00	
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $710.00	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $690.00	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	$0.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	13 - 20 =	0	x $18.00	$0.00
Independent claims	1 - 3 =	0	x $80.00	$0.00
Multiple Dependent Claims **(check if applicable)**.			☒	$270.00
TOTAL OF ABOVE CALCULATIONS =				$1,130.00
☐ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				$0.00
SUBTOTAL =				$1,130.00
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				$0.00
TOTAL NATIONAL FEE =				$1,130.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				$0.00
TOTAL FEES ENCLOSED =				$1,130.00
			Amount to be: refunded	$
			charged	$

a. ☒ A check in the amount of **$1,130.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

22850

Surinder Sachar
Registration No. 34,423

(703) 413-3000

Surinder Sachar
SIGNATURE

Norman F. Oblon
NAME

24,618
REGISTRATION NUMBER

6-22-01
DATE

Description

Monoclonal Antibody Against Apolipoprotein A-I

Technical Field

The present invention relates to a monoclonal antibody against a specific human apolipoprotein A-I (hereinafter referred to as "apoA-I"); a method of immunologically assaying a specific apoA-I by use of the antibody; and an immunological assay reagent containing the antibody.

Background Art

ApoA-I is a predominant apoprotein that constitutes HDL and plays an important role in reserve cholesterol transportation from peripheral cells to the liver (Philips M. C. *et al.*, *Biochem. Biophys. Acta*, 906: p. 223 (1987)). Therefore, apoA-I assay is performed in the diagnosis of arteriosclerosis.

In recent years, researchers have elucidated that an HDL containing apoA-I but containing no apolipoprotein A-II (hereinafter referred to as "apoA-II") (Ishizuka *et al.*, "*Igaku to Yakugaku*," Vol. 39, No. 5, p. 1041, 1988) exhibits a stronger effect of pulling cholesterol from cells as compared with an HDL containing both apoA-I and apoA-II, and that an apoA-I not binding to a lipid and an apoA-I which is in the form of small particles and occurs in preβ1-HDL containing a small amount of lipid (T. Miida *et al.*,

Biochemistry, 29: p. 10469 (1990)) play an important role in reverse cholesterol transport system from cells. Accordingly, assaying these specific apoA-I's is of increased importance. Among HDLs containing apoA-I but containing no apoA-II, preβ1-HDL pulls cholesterol from peripheral cells through interaction specific to the cell surface (Fielding, C. *et al., Lipid Res.,* 36: p. 211-228 (1995)), and its action is more effective than that of HDL. Thus, preβ1-HDL is particularly attracting researchers' attention.

However, since no antibody that selectively reacts with a specific apoA-I has been found, the target apoA-I must be isolated from other apoA-I's through a method such as electrophoresis or immune precipitation. Thus, a specific apoA-I cannot be assayed in a simple manner.

Disclosure of the Invention

In view of the foregoing, the present inventors have carried out extensive studies and have successfully obtained a monoclonal antibody which specifically reacts with a certain species of apoA-I. The inventors have found that, by use of the monoclonal antibody, apoA-I's such as the aforementioned apoA-I not binding to a lipid and apoA-I composing preβ1-HDL can be assayed accurately in a simple manner, thereby enabling more accurate diagnosis of lipid metabolism disorder to be performed. The present invention has been accomplished on the basis of this finding.

Accordingly, the present invention provides a

monoclonal antibody reacting specifically with (1) an apoA-I occurring in HDL which contains no apoA-II and has a molecular weight of 150,000 or less and (2) an apoA-I not binding to a lipid.

The present invention also provides a hybridoma for producing the monoclonal antibody.

The present invention also provides a method of immunologically assaying apoA-I, characterized by reacting the monoclonal antibody with a specimen.

Furthermore, the present invention provides a reagent for assaying an apoA-I containing the monoclonal antibody.

Brief Description of the Drawings

Fig. 1 shows western blotting profiles (electrophoresis) indicating the specificity of the antibody of the present invention. Fig. 2 is a graph showing the specificity of the antibody of the present invention as obtained by ELISA. Fig. 3 contains graphs showing reactivity of the antibody of the present invention to fractions obtained through gel filtration.

Fig. 4 shows two-dimensional electrophoresis profiles showing the specificity of the antibody of the present invention. Fig. 5 is a graph showing dilution proportionality of the antibody of the present invention during ELISA. Fig. 6 contains charts showing the results of preβ1-HDL level assay on clinical specimens. Fig. 7 shows the preβ1-HDL levels as determined after heating of the clinical specimens. Fig. 8

shows percent decreases in preβ1-HDL level of clinical specimens after heating.

Best Modes for Carrying Out the Invention

The monoclonal antibody of the present invention can be produced, for example, in the following manner.

Purified apoA-I or a lipoprotein containing apoA-I is used as an immunogen. No particular limitation is imposed on the animals for immunization, and animals such as mice and rats are generally used. Immunization can be performed through a routine method. For example, there may be employed a method in which a suspension of an immunogen in a commonly employed buffer or physiological saline, or a mixture of the suspension and a replenisher such as the complete Freund's adjuvant is administered to an animal subcutaneously, intracutaneously, or intraperitoneally so as to perform primary immunization, and the immunization is repeated in accordance with needs. The administration dose of the immunogen——appropriately determined depending on the route of administration and the species of animal——is typically controlled to approximately 10 μg to 1 mg per administration. The immunocompetent cells to be used for cell fusion are preferably spleen cells collected 3-4 days after completion of immunization. Myeloma cells that serve as parent cells to be fused with the aforementioned immunocompetent cells may be of any known cell line that has already been established. Examples of the cell line include mouse cell lines such as

NS1 (P3/NSI/I-Ag4444-1) [*Eur. J. Immunol.* 6:511-519 (1976)]; SP2/O-Ag14 [*Nature* 276:269 (1978)]; P3X63-Ag8.653 [*J. Immunol.* 123:1548 (1979)]; and P3X63-Ag8U.1 [*Curr. Top. Microbiol. Immunol.* 81:1 (1978)] and rat cell lines such as Y3-Ag1.2.3 [*Nature* 277:131-133 (1979)]; YB2/O (YB2/3HL/P2.G11.16Ag.20) [*Methods Enzymol.* 73B:1 (1981)], and any of these can be used. In order to perform cell fusion, typically employed poly(ethylene glycol) (PEG), Sendai virus (HVJ), or a similar material can be used. Cell fusion may be performed in a typically employed manner. Specifically, immunocompetent cells are used in a total amount of approximately 1-10 times the amounts of the bone marrow cells, and poly(ethylene glycol) having an average molecular weight of 1,000-6,000 is used at a concentration of 30-60%. Poly(ethylene glycol) is added dropwise to pellets formed of a mixture of the immunocompetent cells and the bone marrow cells, and the resultant mixture is mixed. The target hybridoma may be selected by use of a typical culture medium such as an HAT medium (i.e., a medium containing hypoxanthine, aminopterin, and thymidine).

After completion of culturing in the HAT medium, the yielded hybridoma is subjected to typical limiting dilution analysis for retrieval of a target-antibody-producing strain and obtaining a monoclone. In the retrieval of the target-antibody-producing strain, ELISA, RIA, or a similar assay can be employed, whereby a hybridoma producing an antibody which reacts specifically with a specific apoA-I can be selected.

The monoclonal antibody of the present invention may be selected through a method as described below.

Firstly, monoclonal antibodies contained in the supernatant of the culture medium are immobilized by use of an immobilizer such as an anti-mouse-IgG antibody, and, subsequently, a lipoprotein-containing mixture such as blood plasma is caused to react. Next, an anti-apoA-I antibody labeled with a labeling agent such as an enzyme or a similarly labeled antibody against apoA-II is caused to react, to thereby select a monoclonal antibody which reacts specifically with the anti-apoA-I antibody system but does not react with the anti-apoA-II antibody system.

Examples of the hybridoma producing such a monoclonal antibody include hybridoma 55201, which the present inventors previously found. Hybridoma 55201 was deposited as FERM BP-6938 (original depository date: 1998/11/17) to the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (address: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki 305-8566 JAPAN).

The target antibody is produced from the thus-produced antibody-producing hybridoma by means of culturing the hybridoma in a routine manner and separating the antibody from the culture supernatant. Alternatively, the target antibody is obtained by administering the aforementioned hybridoma to a mammal adaptable to the hybridoma and collecting the antibody from ascites.

Monoclonal antibody 55201 produced by hybridoma 55201

is endowed with the following properties:

(A) to react with (1) an apoA-I occurring in HDL which contains no apoA-II and has a molecular weight of 150,000 or less and (2) an apoA-I not binding to a lipid, and

(B) to react with an apoA-I contained in ultracentrifugal fractions of HDL3 and bottom when blood plasma taken from a healthy human is fractionated by means of an ultracentrifuge into five fractions; i.e., VLDL, LDL, HDL2, HDL3, and bottom.

In the aforementioned (A), preβ1-HDL is particularly preferred as the (1) HDL which contains no apoA-II and has a molecular weight of 150,000 or less.

By use of the aforementioned antibody, a specific apoA-I contained in a human specimen can be assayed through any conventional immunological assay method. Blood plasma or blood serum is used as the specimen. Examples of the employable immunological assay method include RIA, EIA, and similar methods performed on the basis of a conventional competition method or sandwich method. When these methods are employed, a labeled antibody of the present invention may also be used. Examples of the substance for labeling include enzymes such as peroxidase, alkaline phosphatase, glucoamylase, and β-galactoxidase, and radioactive substances such as ^{125}I, ^{131}I, and tritium. Examples of supports for immobilizing the antibody include a variety of plastic wells and plastic beads.

When assay is performed by ELISA, purified apoA-I is

used as a standard, and quantification can be performed in the following manner. Specifically, a diluted sample is added to an ELISA plate onto which the monoclonal antibody of the present invention is immobilized, to thereby cause reaction. Subsequently, an enzyme-labeled anti-apoA-I polyclonal antibody is caused to react to develop color, and apoA-I contained in the sample is quantitatively determined based on the change in absorbance.

Similar to the case of a typical immunological assay, these assays can be performed at any temperature within 0-40°C.

As described above, the aforementioned (A) apoA-I (1) and/or apoA-I (2) contained in blood plasma or blood serum can be assayed by use of the monoclonal antibody of the present invention. However, assay values of specimens heated at 37°C are considerably low as compared with those non-heated. This indicates that the concentration of the aforementioned (A) apoA-I in blood plasma or blood serum lowers by heating at 37°C. Accordingly, the aforementioned (A) apoA-I contained in a specimen can also be assayed on the basis of this property. Specifically, the aforementioned (A) apoA-I is assayed through the aforementioned immunological assay method before and after heating the specimen, and the percent decrease, or amount of reduction, in assayed value attributable to heating is measured. Thus, the aforementioned (A) apoA-I can also be assayed. The temperature of the specimen is preferably 0-25°C before

heating and 30-40°C after heating. Specimens before heating also include a specimen stored at a temperature as low as 0-10°C.

By employment of these assay methods, the aforementioned (A) apoA-I (1) and/or apoA-I (2) contained in blood plasma or blood serum can be assayed accurately in a simple manner.

Examples

The present invention will next be described in more detail by way of examples, which should not be construed as limiting the invention thereto.

Example 1 (Preparation of monoclonal antibody)

(1) Preparation of hybridoma

Pooled serum of a healthy human was ultracentrifuged, to thereby separate HDL, and the separated HDL was defatted by use of an ethanol-ether mixture and ether. After ether had been completely removed by nitrogen gas, the residue was re-dissolved in an 8M urea solution. The resultant solution was subjected to gel filtration by use of a Sephacryl S200 column (product of Pharmacia). From among separated fractions, fractions containing apoA-I were collected, and the mixture was dialyzed against PBS, to thereby obtain an immunogen. This immuogen and complete Freund's adjuvant (product of GIBCO) were admixed at 1 : 1, to thereby prepare a 0.1 mg/0.1 ml emulsion. The emulsion was subcutaneously administered to a 6-week old female BALB/C mouse six times at

one-week intervals. Two days after completion of final immunization, the spleen was extirpated. Spleen cells obtained from the extirpated spleen and myeloma cells SP2/O-Ag14 were mixed at 6 : 1, and two cell species were fused in the presence of 50% polyethylene glycol 1540 (product of Wako Pure Chemical Industries, Ltd.). The thus-fused cells were suspended in an HAT medium such that the cell concentration was controlled to 2.5×10^6/mL as reduced to spleen cells. The suspension was poured into a 96-well culture plate (product of Corning) at 0.2 mL/well, followed by culturing at 37°C in a 5%CO_2 incubator. After approximately two weeks, a candidate antibody-producing strain was selected, through the ELISA method described hereunder, from a culture supernatant occurring in wells in which hydridoma had been grown. Briefly, IgG contained in each culture supernatant was immobilized on a microplate (product of Nunc) by the mediation of a goat anti-mouse IgG (Fc) antibody (product of Jackson). To the IgG-immobilized plate, a diluted plasma liquid of a healthy human was added so as to cause reaction of a lipoprotein (predominantly containing HDL) containing apoA-I. Subsequently, there was reacted a biotin-labeled anti-apoA-I antibody which had been prepared by biotinylating, with biotin-N-hydroxysuccinimide (product of Zymed), a goat anti-apoA-I antibody obtained by immunization of a goat with apoA-I or a biotin-labeled anti-apoA-II antibody which had been prepared by similarly biotinylating a goat anti-apoA-II antibody obtained by immunization of a goat with apoA-II.

Further, peroxidase-labeled storeptoavidin (product of Zymed) was added for reaction. After these reactions were complete, the resultant product was allowed to develop color with a substrate solution containing o-phenylenediamine (product of Tokyo Kasei). The degree of coloring was measured by means of a microplate reader (A. 492), to thereby select strains which exhibited high reactivity in the system employing the biotin-labeled anti-apoA-I antibody but exhibited no reactivity in the system employing the biotin-labeled anti-apoA-II antibody. The thus-obtained hybridoma was cloned through limiting dilution, to thereby prepare monoclonal antibody hybridoma 55201.

(2) Preparation of monoclonal antibody

To each of 12-week old female BALB/C mice to which pristane (0.5 mL) had been intraperitoneally injected in advance, hybridoma 55201 (cells 0.5×10^6) was intraperitoneally administered two weeks after the above injection of pristane. About 14 days after administration of the hybridoma, ascites was collected and centrifuged, to thereby yield a supernatant. The supernatant was admixed with an equi-amount of a buffer for adsorption (3M NaCl-1.5M glycine-NaOH, pH 8.5), and the mixture was filtered. The resultant filtrate was passed through a protein A column (product of Pharmacia) which had been equilibrated with the buffer for adsorption, to thereby adsorb the antibody onto the column. The adsorbed antibody was eluted by use of a 0.1M citric acid buffer (pH 3.0), to thereby purify the

monoclonal antibody 55201.

Example 2 (Specificity of monoclonal antibody)

(1) Western blotting

In order to confirm that the antibody obtained in Example 1 is in fact an antibody against apoA-I, the antibody was analyzed through western blotting. Specifically, blood serum of a healthy human was subjected to SDS-polyacrylamide gel electrophoresis, and the separated matter was electrically transferred to a PVDF membrane (product of Millipore). The transferred matter was blocked for one hour with PBST (PBS supplemented with 0.05% Tween 20) containing 3% skim milk. Subsequently, the monoclonal antibody 55201 and peroxidase-labeled anti-mouse IgG antibody (product of American Qualex) were reacted as a primary antibody and a secondary antibody, respectively. After the PVDF membrane had been washed with PBST, diaminobenzidine was added as a substrate, to thereby allow to develop color. As shown in Fig. 1, the monoclonal antibody 55201 exhibited a unique band corresponding to an apoA-I of a molecular weight of 28,000, confirming that the antibody is an antibody specific for apoA-I.

(2) ELISA

The monoclonal antibody (55201) obtained in Example 1 was diluted with 20mM phosphate buffered saline (PBS; pH 7.2), to thereby adjust the concentration to 3 μg/mL. The diluted antibody was added to a 96-well ELISA plate (product of Nunc)

at 50 μL/well and incubated at 4°C for one night. The plate was washed three times with PBS, and, subsequently, a blocking liquid (PBS containing 1% BSA) was added at 100 μL/well, to thereby carry out blocking for one hour. After removal of the blocking liquid, blood plasma of a healthy human diluted by the blocking liquid was added at 50 μL/well, and incubation was carried out for one hour at room temperature. The plate was washed three times with the blocking liquid, and, subsequently, a biotin-labeled goat anti-apoA-I antibody or a biotin-labeled anti-apoA-II antibody was added at 50 μL/well, and incubation was carried out for one hour at room temperature. In a similar manner, the plate was washed three times with the blocking liquid, and, subsequently, peroxidase-labeled storeptoavidin was added, followed by incubation for 30 minutes at room temperature. The plate was washed again three times with the blocking liquid, and, subsequently, a peroxidase substrate solution was added at 50 μL/well. Ten minutes after addition of the substrate solution, 1.5N sulfuric acid was added at 50 μL/well, and absorbance at 492 nm was measured.

Fig. 2 shows the results. As is clear from Fig. 2, the monoclonal antibody 55201 does not react with HDL containing apoA-II, but reacts specifically with HDL containing apoA-I alone.

(3) Reactivity with respect to fractions separated through gel filtration

In order to study the specificity of the antibody

obtained in Example 1, blood plasma of a healthy human was separated through gel filtration, and the reactivity of the antibody to each separated fraction was investigated. Specifically, blood plasma of a healthy human was separated by means of a Pharmacia FPLC system in which four gel filtration columns (TSK-GEL G3000SW, 7.5 mmID × 60 cm (×2); G3000SW, 7.5 mmID × 30 cm (×1); and Pharmacia Superdex 200 HR10/30 (×1)) were connected. The apoprotein concentration of each fraction was measured and the reactivity of the monoclonal antibody to each fraction was compared through the below-described ELISA method. The monoclonal antibody 55201 obtained in Example 1 was diluted with 20mM phosphate buffered saline (PBS; pH 7.2), to thereby adjust the concentration to 3 μg/mL. The diluted antibody was added to a 96-well ELISA plate (product of Nunc) at 50 μL/well and incubated at 4°C for one night. The plate was washed three times with PBS, and, subsequently, a blocking liquid (PBS containing 1% BSA) was added at 100 μL/well, to thereby carry out blocking for one hour. After removal of the blocking liquid, each fraction diluted by the blocking liquid or purified apoA-I was added at 50 μL/well, and incubation was carried out for one hour at room temperature. The plate was washed three times with the blocking liquid, and, subsequently, a peroxidase-labeled goat anti-apoA-I antibody which had been obtained by labeling a goat anti-apoA-I antibody, yielded by immunizing a goat with apoA-I, through a periodic acid method, was added at 50 μL/well, and incubation

was carried out for one hour at room temperature. In a similar manner, the plate was washed three times with the blocking liquid, and, subsequently, a peroxidase substrate solution was added at 50 μL/well. Ten minutes after addition of the substrate solution, 1.5N sulfuric acid was added at 50 μL/well, and absorbance at 492 nm was measured. Based on purified apoA-I as a standard, the amount of apoA-I in each fraction was calculated. The results are shown in Fig. 3 (lower). Each of apoproteins; i.e., apoA-I, apoA-II, and apo-E, was assayed through ELISA by use of a polyclonal antibody obtained by immunizing a goat with each apoprotein and a peroxidase-labeled antibody thereof obtained through a periodic acid method. The results are shown in Fig. 3 (upper). As is clear from Fig. 3 (lower), the monoclonal antibody 55201 predominantly reacts specifically with an apoA-I contained in HDL which occurs in blood plasma and has a molecular weight of 67,000 or less, or with an apoA-I not binding to a lipid.

(4) Reactivity with respect to ultracentrifuged fractions

In order to study the specificity of the monoclonal antibody 55201 obtained in Example 1, blood plasma of a healthy human (30 mL) was separated by means of an ultracentrifuge (product of Hitachi) into five fractions; i.e., VLDL, LDL, HDL2, HDL3, and bottom, so as to determine which fraction contains particles reactive with the antibody 55201. Specifically, the amount of apoA-I in each separated fraction was measured through ELISA as employed in Example

2(3), with reference to purified apoA-I as a standard. Table 1 shows the results. As shown in Table 1, fractions VLDL, LDL, and HDL2 contain substantially no plasma component reactive with the monoclonal antibody 55201, and fractions HDL3 and bottom contain such a reactive plasma component. The results indicate that the antibody 55201 does not react with an apoA-I contained in a fraction of low specific gravity such as HDL2, but reacts with apoA-I contained in fractions of high specific gravity; i.e., HDL3 and bottom.

Table 1

Fractions	Specific gravity	apoA-I (μg)*
VLDL	<1.006	0
LDL	1.006-1.063	12
HDL2	1.063-1.125	24
HDL3	1.125-1.21	1296
Bottom	1.21<	1785

* the amount of apoA-I in particles reactive with the antibody 55201

(5) Reaction with preβ1-HDL

In order to study the specificity of the monoclonal antibody 55201 obtained in Example 1, analysis through non-denaturing two-dimensional electrophoresis was carried out. Specifically, the monoclonal antibody 55201 or normal mouse IgG was added to blood plasma of a healthy human, and each sample was subjected to 0.75% agarose gel electrophoresis. A fragment was cut from the agarose gel, and the cut agarose gel fragment was subjected to polyacrylamide gel electrophoresis. The separated matter was electrically transferred to nitrocellulose membrane (product of Millipore)

and reacted with a ^{125}I-labeled goat anti-apoA-I antibody. The changes observed in the HDL subfractions were compared through autoradiography. The results are shown in Fig. 4. In contrast with the subfraction using a normal mouse IgG system serving as a control, spots attributed to preβ1-HDL disappeared and spots possibly corresponding to formation of a complex of IgG and preβ1-HDL in monoclonal antibody 55201-added system were observed, whereas spots attributed to HDLs other than preβ1-HDL were unchanged. The results indicate that the monoclonal antibody 55201 reacts specifically with preβ1-HDL.

(6) preβ1-HDL level after heating at 37°C

In order to study the specificity of the monoclonal antibody 55201 obtained in Example 1, blood plasma of a healthy human (0.2 mL) which had been stored at 4°C was poured into microtubes, followed by incubation at 37°C for two hours, to thereby investigate the effect on reactivity with the antibody. Specifically, the preβ1-HDL concentration in blood plasma was measured, before and after incubation, through ELISA as employed in Example 2(3) with reference to purified apoA-I as a standard. Table 2 shows the results. As is clear from Table 2, the reactivity lowers considerably after incubation at 37°C; i.e., the level of preβ1-HDL in blood is lowered by heating.

Table 2

	Level measured (μg/mL)	
	Before incubation	after incubation
Specimen 1	19.9	2.1
Specimen 2	20.1	2.7
Specimen 3	16.9	4.0
Specimen 4	20.7	5.0
Specimen 5	21.8	2.2

Example 3 (Method of measurement)

By use of the antibody obtained in Example 1, the preβ1-HDL concentration in human blood plasma (three specimens) was measured. Specifically, the preβ1-HDL concentration in human blood plasma was measured through ELISA as employed in Example 2(3) with reference to purified apoA-I as a standard. As shown in Fig. 5, all three specimens exhibit favorable dilution proportionality, indicating the feasibility of measuring the preβ1-HDL concentration.

Example 4 (Measurement of clinical specimens)

In order to study the clinical significance of preβ1-HDL measurement confirmed in Example 3, blood plasma specimens of 39 hyperlipidemia patients and blood specimens of 11 healthy humans were provided, with all specimens stored at 4°C. The preβ1-HDL concentration in each blood plasma specimen before and after heating at 37°C was measured through ELISA as employed in Example 2(3). In addition, as a control, the total apoA-I concentration in blood plasma——obtained through a conventional technique——was measured through immunoturbidimetry by use of blood plasma

specimens before heating at 37°C.

Fig. 6 shows the total apoA-I concentration and preβ1-HDL concentration of blood plasma specimens before heating at 37°C. Although no clear difference was observed between the patients and healthy humans in terms of the total apoA-I concentration, considerable difference was observed between the patients and healthy humans in terms of the preβ1-HDL concentration.

Fig. 7 shows the preβ1-HDL concentration after heating. Considerable difference was observed between the patients and healthy humans in terms of the average preβ1-HDL concentration; i.e., 3.8 μg/mL for healthy humans and 12.2 μg/mL for the patients.

Fig. 8 shows the percent reduction of preβ1-HDL concentration before heating to that after heating. Considerable difference was observed between the patients and healthy humans in terms of the average percent reduction; i.e., 79.1% for healthy humans and 68.5% for the patients.

The above-described results indicate that measuring the preβ1-HDL concentration before and after heating and measuring reduction in the concentration after heating or the reduction ratio thereof provide useful indices showing lipid metabolism disorders.

Industrial Applicability

By use of the antibody of the present invention, which reacts specifically with a specific apoA-I, a specific apoA-I

contained in human body fluid can be measured, providing a novel index for lipid metabolism disorder and the like.

*****

Claims

1. (amended) A monoclonal antibody which reacts specifically with (1) a human apolipoprotein A-I occurring in HDL which contains no human apolipoprotein A-II and has a molecular weight of 150,000 or less and (2) a human apolipoprotein A-I not binding to a lipid, but does not react with (1) HDL containing apoA-II, (2) VLDL, (3) LDL, or (4) HDL2, and the reactivity of the monoclonal antibody heated at 37°C to blood plasma or blood serum is low as compared with that of the non-heated monoclonal antibody.

2. The monoclonal antibody as described in claim 1, wherein the HDL (1) which contains no human apolipoprotein A-II and has a molecular weight of 150,000 or less is preβ1-HDL.

3. A hybridoma for producing the monoclonal antibody as recited in claim 1 or 2.

4. An immunoassay method for a human apolipoprotein A-I, characterized by reacting the monoclonal antibody as recited in claim 1 or 2 with a specimen.

5. The method as described in claim 4, which is performed through RIA or EIA.

6. The method as described in claim 4 or 5, wherein measurement is performed before and after heating the specimen, and the amount of reduction or percent reduction after heating is determined.

7. A reagent for assaying a human apolipoprotein A-I containing the monoclonal antibody as recited in claim 1 or 2.

8. The reagent according to claim 7, which is for RIA or EIA.

FIG. 1

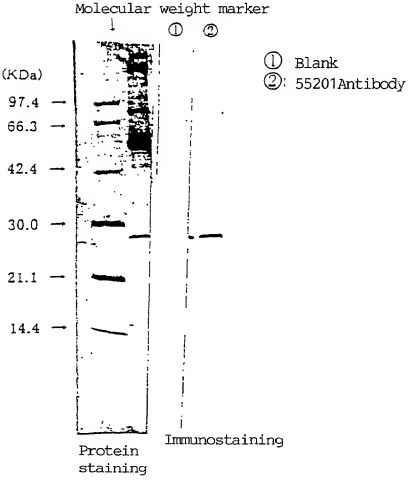

Molecular weight marker
① ②
(KDa)
97.4
66.3
42.4
30.0
21.1
14.4
① Blank
②: 55201Antibody
Protein staining
Immunostaining

FIG. 2

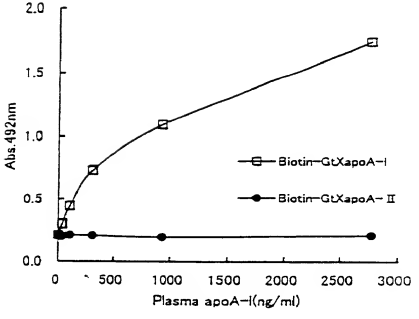

2.0
1.5
1.0
0.5
0.0
Abs.492nm
0
500
1000
1500
2000
2500
3000
Plasma apoA-I(ng/ml)
Biotin-GtXapoA-I
Biotin-GtXapoA-II

FIG. 3

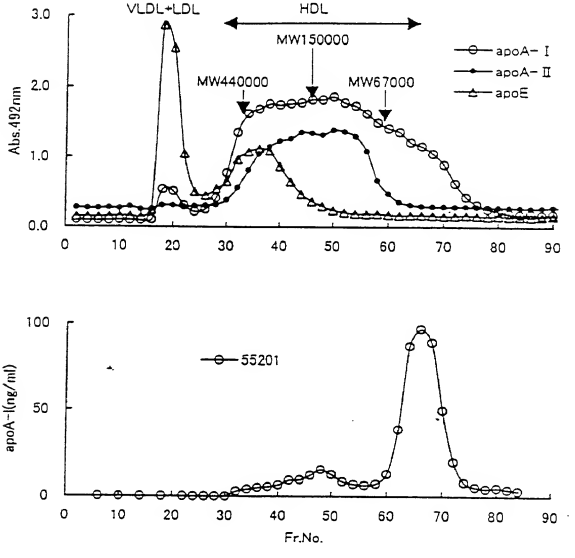

VLDL+LDL
HDL
MW150000
MW440000
MW67000
apoA-Ⅰ
apoA-Ⅱ
apoE
Abs.492nm
3.0
2.0
1.0
0.0
0
10
20
30
40
50
60
70
80
90
100
50
0
55201
apoA-Ⅰ(ng/ml)
Fr.No.

FIG. 4

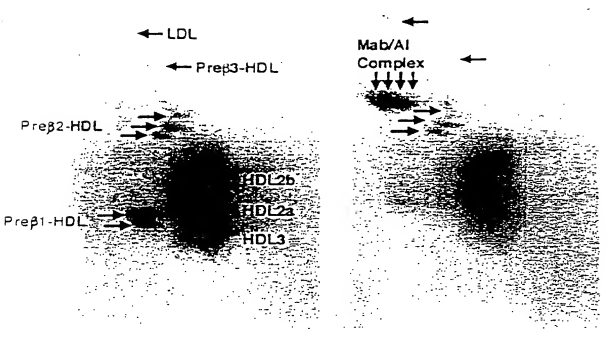

A: Control
B: Mab55201
LDL
Preβ3-HDL
Preβ2-HDL
HDL2b
HDL2a
HDL3
Preβ1-HDL
Mab/AI
Complex

FIG. 5

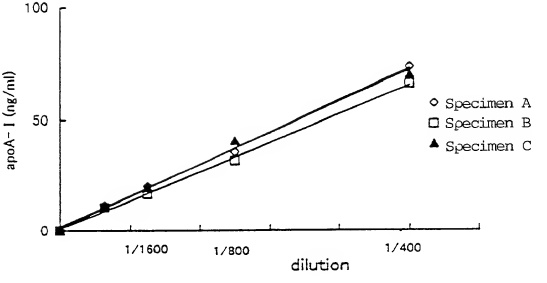
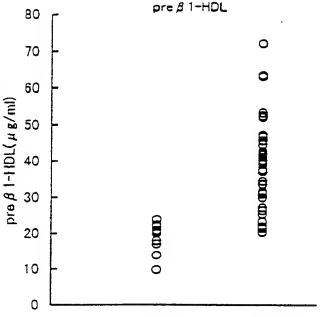
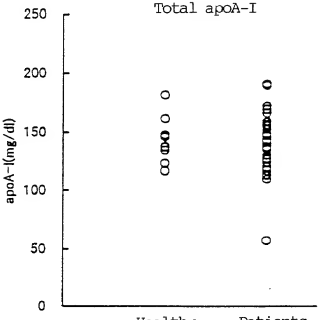

100
50
0
apoA- I (ng/ml)
1/1600
1/800
1/400
dilution
Specimen A
Specimen B
Specimen C

FIG. 6

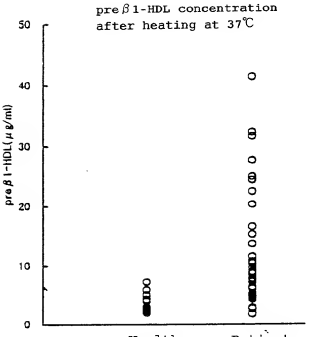


	Healthy humans	Patients
n	11	39
mean	18.9	38.6
SD	4.1	12.0



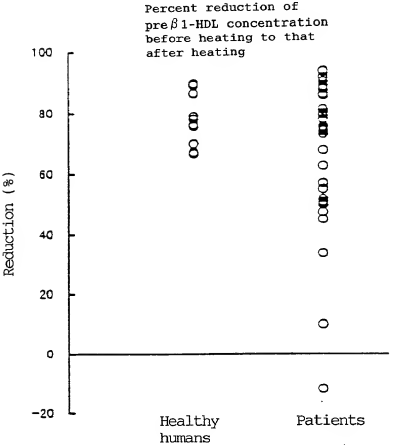
	Healthy humans	Patients
n	11	39
mean	144	142
SD	18	25

FIG. 7



	Healthy humans	Patients
n	11	39
mean	3.8	12.2
SD	1.7	9.5

FIG. 8



	Healthy humans	Patients
n	11	39
mean	79.1	68.5
SD	8.9	22.8

Declaration and Power of Attorney For Patent Application

特許出願宣言書及び委任状

Japanese Language Declaration

日本語宣言書

下記の氏名の発明者として、私は以下の通り宣言します。

As a below named inventor, I hereby declare that:

私の住所、私書箱、国籍は下記の私の氏名の後に記載された通りです。

My residence, post office address and citizenship are as stated next to my name.

下記の名称の発明に関して請求範囲に記載され、特許出願している発明内容について、私が最初かつ唯一の発明者（下記の氏名が一つの場合）もしくは最初かつ共同発明者（下記の名称が複数の場合）であると信じています。

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled.

アポリポ蛋白質 A-I に対するモノクローナル抗体

MONOCLONAL ANTIBODY AGAINST APOLIPOPROTEIN A-I

上記発明の明細書は、

the specification of which

☐ 本書に添付されています。

☐ is attached hereto.

☒ 12月 17日に提出され、米国出願番号または特許協定条約国際出願番号を 07106 とし、
(該当する場合) 9月 12日に訂正されました。

☒ was filed on December 17, 1999
as ~~United States Application Number~~ or
PCT International Application Number
PCT/JP99/07106 and was amended on
Sept.12,2000 (if applicable).

私は、特許請求範囲を含む上記訂正後の明細書を検討し、内容を理解していることをここに表明します。

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

私は、連邦規則法典第37編第1条56項に定義されるとおり、特許資格の有無について重要な情報を開示する義務があることを認めます。

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

Japanese Language Declaration
(日本語宣言書)

私は、米国法典第35編119条（a）-（d）項又は365条（b）項に基づき下記の、米国以外の国の少なくとも一カ国を指定している特許協力条約365（a）項に基づく国際出願、又は外国での特許出願もしくは発明者証の出願についての外国優先権をここに主張するとともに、優先権を主張している、本出願の前に出願された特許または発明者証の外国出願を以下に、枠内をマークすることで、示しています。

I hereby claim foreign priority under Title 35, United States Code, Section 119 (a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s) 外国での先行出願			Priority Claimed 優先権主張	
10-364295	Japan	22/12/1998	☒	☐
(Number) (番号)	(Country) (国名)	(Day/Month/Year Filed) (出願年月日)	Yes はい	No いいえ
			☐	☐
(Number) (番号)	(Country) (国名)	(Day/Month/Year Filed) (出願年月日)	Yes はい	No いいえ

私は、第35編米国法典119条（e）項に基づいて下記の米国特許出願規定に記載された権利をここに主張いたします。

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

(Application No.) (出願番号)	(Filing Date) (出願日)	(Application No.) (出願番号)	(Filing Date) (出願日)

私は、下記の米国法典第35編120条に基づいて下記の米国特許出願に記載された権利、又は米国を指定している特許協力条約365条（c）に基づく権利をここに主張します。また、本出願の各請求範囲の内容が米国法典第35編112条第1項又は特許協力条約で規定された方法で先行する米国特許出願に開示されていない限り、その先行米国出願書提出日以降で本出願書の日本国内または特許協力条約国際提出日までの期間中に入手された、連邦規則法典第37編1条56項で定義された特許資格の有無に関する重要な情報について開示義務があることを認識しています。

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of application.

(Application No.) (出願番号)	(Filing Date) (出願日)	(Status: Patented, Pending, Abandoned) (現況：特許許可済、係属中、放棄済)
(Application No.) (出願番号)	(Filing Date) (出願日)	(Status: Patented, Pending, Abandoned) (現況：特許許可済、係属中、放棄済)

私は、私自信の知識に基づいて本宣言書中で私が行なう表明が真実であり、かつ私の入手した情報と私の信じるところに基づく表明が全て真実であると信じていること、さらに故意になされた虚偽の表明及びそれと同等の行為は米国法典第18編第1001条に基づき、罰金または拘禁、もしくはその両方により処罰されること、そしてそのような故意による虚偽の声明を行なえば、出願した、又は既に許可された特許の有効性が失われることを認識し、よってここに上記のごとく宣誓を致します。

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Japanese Language Declaration
(日本語宣言書)

委任状：私は下記の発明者として、本出願に関する一切の手続きを米特許商標局に対して遂行する弁理士または代理人として、下記の者を指名いたします。
(弁護士、または代理人の指名及び登録番号を明記のこと)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: *(list name and registration number)*

30

Norman F. Oblon, Reg. No. 24,618; Marvin J. Spivak, Reg. No. 24,913; C. Irvin McClelland, Reg. No. 21,124; Gregory J. Maier, Reg. No. 25,599; Arthur I. Neustadt, Reg. No. 24,854; Richard D. Kelly, Reg. No. 27,757; James D. Hamilton, Reg. No. 28,421; Eckhard H. Kuesters, Reg. No. 28,870; Robert T. Pous, Reg. No. 29,099; Charles L. Gholz, Reg. No. 26,395; William E. Beaumont, Reg. No. 30,996; Robert F. Gnuse, Reg. No. 27,295; Jean-Paul Lavalleye, Reg. No. 31,451; Stephen G. Baxter, Reg. No. 32,884; Robert W. Hahl, Reg. No. 33,893; Richard L. Treanor, Reg. No. 36,379; Steven P. Weihrouch, Reg. No. 32,829; John T. Goolkasian, Reg. No. 26,142; Richard L. Chinn, Reg. No. 34,305; Steven E. Lipman, Reg. No. 30,011; Carl E. Schlier, Reg. No. 34,426; James J. Kulbaski, Reg. No. 34,648; Richard A. Neifeld, Reg. No. 35,299; J. Derek Mason, Reg. No. 35,270; Surinder Sachar, Reg. No. 34,423; Christina M. Gadiano, Reg. No. 37,628; Jeffrey B. McIntyre, Reg. No. 36,867; Paul E. Rauch, Reg. No. 38,591; William T. Enos, Reg. No. 33,128 and Michael E. McCabe, Jr., Reg. No. 37,182, with full powers of substitution and revocation.

書類送付先

Send Correspondence to:

OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C.
FOURTH FLOOR
1755 JEFFERSON DAVIS HIGHWAY
ARLINGTON, VIRGINIA 22202 U.S.A.

直接電話連絡先：（名前及び電話番号）

Direct Telephone Calls to: (name and telephone number)

(703) 413-3000

単独発明者または第一の共同発明者の氏名 宮崎　修一	Full name of sole or first joint inventor Osamu MIYAZAKI
発明者の署名　日付 Osamu Miyazaki　June 1, 2001	Inventor's signature　Date
住所　319-1112　日本国茨城県那珂郡東海村村松２１１７ 第一化学薬品株式会社診断薬研究所内	Residence　C/O Daiichi Pure Chemicals Co., Ltd. Tokai Laboratories 2117, Muramatsu, Tokaimura, Naka-gun, Ibaraki 319-1112 JAPAN
国籍 日本国	Citizenship JAPANESE　JPX
郵便の宛先　住所に同じ	Post Office Address SAME AS ABOVE
第二の共同発明者の氏名 深町　勇	Full name of second joint inventor, if any Isamu FUKAMACHI
第二の共同発明者の署名　日付 Isamu Fukamachi　June 1, 2001	Second joint Inventor's signature　Date
住所　319-1112　日本国茨城県那珂郡東海村村松２１１７ 第一化学薬品株式会社診断薬研究所内	Residence　C/O Daiichi Pure Chemicals Co., Ltd. Tokai Laboratories 2117, Muramatsu, Tokaimura, Naka-gun, Ibaraki 319-1112 JAPAN
国籍 日本国	Citizenship JAPANESE　JPX
郵便の宛先　住所に同じ	Post Office Address SAME AS ABOVE

（第三以降の共同発明者についても同様に記載し、署名すること）

(Supply similar information and signature for third and subsequent joint inventors.)